DEVELOPMENT OF AN ANALYTICAL PROCEDURE USING 1-NITROPYRENE METABOLITES AS URINARY BIOMARKERS OF EXPOSURE TO DIESEL PARTICULATE MATTER

An Undergraduate Research Scholars Thesis

By

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ABSTRACT

Development of an analytical procedure using 1-nitropyrene metabolites as urinary biomarkers of exposure to diesel particulate matter. (May 2014)

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Diesel exhaust particulate matter (DPM) is a major contributor to air pollution and has been recently classified as a group 1 human carcinogen. The combustion of diesel produces a variety of compounds including polycyclic aromatic hydrocarbons (PAHs) and nitrated polycyclic aromatic hydrocarbons (NPAHs). Considerable research has examined the effects of PAHs and NPAHs on human health. This research is limited by the lack of reliable biomarkers of exposure. Biomarkers of exposure allow for the accurate quantification of internalized xenobiotics. The current study demonstrates a highly sensitive analytical method for the quantification of urinary metabolites of 1-nitropyrene (1-NP), the most abundant NPAH present in DPM mixtures. Using liquid chromatography with tandem mass spectrometry (LC-MS/MS), the hydroxyl-1-nitropyrene metabolites (3-, 6-, and 8-OHNP) and hydroxyl-*N*-acetyl-1-aminopyrene metabolites (3-, 6-, and 8-OHNAAP) were synthesized and identified based on retention times and MS/MS spectra. The results of this study provide a sensitive measure of individual exposure to DPM which will facilitate future exposure assessment studies in vulnerable and susceptible study populations.

DEDICATION

I dedicate this thesis to my parents, Mark and Maria Pulczinski who have been a source of support, love and sound advice throughout my life and especially during my college career.

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This thesis would not have been possible without the help and expertise from many individuals. I sincerely thank Dr. Natalie Johnson for her continued support during the course of this study, without which this thesis would not have been possible. Working in her lab has been an enriching experience and has opened my eyes to toxicology as well as given me focus on my goals beyond my undergraduate degree. I would also like to thank Dr. Timothy Phillips and his lab, Dr. Thomas McDonald and Charlie Villamaria who all have offered support, time, advice and encouragement throughout this process. Lastly, I would like to thank the Undergraduate Research Scholars program which made this thesis possible.

NOMENCLATURE

1-AP	1-aminopyrene
1-NP	1-nitropyrene
DPM	diesel particulate matter
OHNAAP	hydroxy-*N*-acetyl-1-aminopyrene
OHNP	hydroxy-1-nitropyrene
Tmax	time of maximum excretion

CHAPTER I

INTRODUCTION

Air pollution is a significant environmental issue and contributes greatly to worldwide morbidity and mortality. In fact, approximately 7 million deaths are attributed to air pollution exposure annually (World Health Organization, 2014a). Air pollution is a broad term that encompasses any form of air contamination, whether physical, chemical, or biological, that alters the natural composition and properties of the atmosphere (World Health Organization, 2014b). Of particular concern to human health are submicron-sized suspended particles called particulate matter. Particulate matter (PM) is classified by the aerodynamic diameter of the particle. Particles with a diameter between 10 μm and 2.5 μm are designated coarse particulate matter or $PM_{10.}$ Particles under 2.5 μm are designated $PM_{2.5}$ or fine particulate matter. Specifically, PM is responsible for over 3 million deaths annually and been linked to illnesses such as asthma, heart disease, and lung disease, including lung cancer (Lim et al., 2013; World Health Organization, 2013b). The incomplete combustion of fossil fuels is a major source of particulate matter and diesel engine exhaust in particular has been identified as significant contributor. Recently, diesel engine exhaust was classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) (Benbrahim-Tallaa et al., 2012; International Agency for Research on Cancer, 2012). This designation indicates there is adequate scientific evidence linking exposure to diesel exhaust to lung cancer and potentially bladder cancer in humans. Due to the widespread use of diesel engines for personal and public transit, generators, shipping, agriculture, and industry, millions of people are regularly exposed to diesel engine exhaust. In the past decades imposed standards in the U.S. have led to a sustained decrease in PM levels stemming from traffic

emissions (Benbrahim-Tallaa et al., 2012). Still, populations living in close proximity to major transit routes, those regularly exposed to non-road emissions (e.g., less regulated diesel powered machinery), or living in developing countries that lack emission standards or poorly enforce emissions regulations are at high risk for exposure.

Exposure during important periods of development (i.e., prenatal and early postnatal) can have lasting effects. Specifically, air pollution has been implicated with low birth weight, respiratory illness and cognitive impairment. Particulate matter exposure in children has been shown to result in lung impairment and reduced lung growth (World Health Organization, 2011). Despite the pervasive use of diesel engines and the hazards posed by DPM exposure, there are limitations in the current exposure assessment strategies making it difficult to study the health effects of DPM exposure. There are several variables that make the study of diesel exhaust particulate matter challenging. The composition of diesel engine exhaust is variable, factors including the age and type of fuel, the age and maintenance of the engine and the presence of an emission control system all affect the compounds present in exhaust (Benbrahim-Tallaa et al., 2012). Additionally, many of the components of diesel engine exhaust exist in the environment due to other sources. Current methods to measure exposure to diesel exhaust often rely on PM measurements from stationary monitors. While sophisticated modeling techniques employing time-activity and geographic information system data have aided in accurately predicting PM levels at people's residential addresses, estimations of exposure level fail to quantify individual exposure levels (Scheepers et al., 2003). A biomarker of exposure to DPM measured in an easily collected biospecimen (blood or urine) would provide the means to identify individuals at greatest risk in biomonitoring studies and serve as accurate measure of exposure in epidemiologic studies investigating health effects resulting from exposure. Furthermore,

biomarkers may be used as intermediate endpoints to monitor the efficacy of interventions or assess the impact of regulations on diesel emissions.

Several biomarkers have been proposed, including nitrated polycyclic aromatic hydrocarbons (NPAHs). NPAHs are produced during the incomplete combustion of fossil fuels, including diesel. 1-Nitropyrene is the most common NPAH product of diesel engine exhaust (Bamford et al., 2003; Rosenkranz, 1982) and is believed to contribute greatly to the overall carcinogenicity of diesel engine exhaust. 1-NP has been identified as genotoxic and mutagenic in several *in vivo* and *in vitro* models and is classified as a Group 2B carcinogen by the IARC, a substance possibly carcinogenic to humans (International Agency for Research on Cancer, 1989; National Toxicology Report, 1996; Salmeen et al., 1982; Schuetzle et al., 1982). Since 1-NP is not formed through the atmospheric reaction of pyrene with hydroxyl or nitrate radicals it has the potential to serve as a valuable biomarker (Atkinson & Arey, 1994; Kielhorn et al., 2003). This aspect greatly limits the chance that exposure will have occurred due to non-combustion sources. Furthermore, detection is facilitated by the fact that 1-NP is a major compound in DPM, which translates into less biospecimen needed to accurately detect and quantify exposure. Finally, the metabolites of 1-NP have been well studied in a variety of animal and cell models. This previous research has established the routes of 1-NP metabolism and identified the major metabolites.

1-Nitropyrene Chemistry

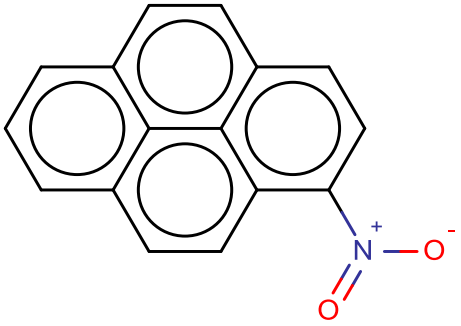

Figure 1. Structure of 1-Nitropyrene

1-Nitropyrene (1-NP) belongs to a class of chemicals called polycyclic aromatic hydrocarbons (PAHs). 1-NP is composed of four carbon rings with a nitro functional group. The molecular formula is $C_{16}H_9NO_2$, and the molecular weight is 247.3 Daltons (International Agency for Research on Cancer, 1989). 1-NP is formed during combustion, specifically when pyrene reacts with atmospheric nitrogen during incomplete combustion of organic compounds; this differs from atmospheric pyrene radical reactions which form 2-nitropyrene or 4-nitropyrene (Atkinson & Arey, 1994; Kielhorn et al., 2003). 1-NP has been identified in the gases of aluminum smelters, wood stoves, certain fried food, and coal power plants, with a major source being the combustion of diesel. 1-NP is degraded by photolysis as well as by ozone, with a rather rapid photolysis half-life of 36 minutes and an half-life of 29 hours in the presence of 0.2 ppm O_3 (Kielhorn et al., 2003). 1-NP present in the atmosphere also varies greatly based on temperature, time of day, and season with greater concentrations generally observed during the winter months, which is thought to be due to an increased use of fossil fuels for heating during the cooler months (Kielhorn et al., 2003). 1-NP concentrations can also fluctuate greatly over the course of a day, with observed peaks coinciding with times of greater traffic volume (Hayakawa et al., 1999; Kielhorn et al., 2003).

1-Nitropyrene Metabolism

Inhalation is a major route of exposure to 1-nitropyrene. In rat models the majority of 1-NP is rapidly cleared by the upper respiratory tract and absorbed into the bloodstream. Then it enters the gastrointestinal tract and is further transported to the liver where it is metabolized and excreted in bile (Medinsky et al., 1985). However, a portion of 1-NP is reabsorbed, which is believed to be facilitated by intestinal bacteria (Medinsky et al., 1985). Metabolism occurs in two phases. Phase I is initiated in one of two ways, nitroreduction or oxidation. Both of these pathways are biologically significantly as metabolites formed via nitroreduction and oxidation can react with DNA forming DNA adducts (Howard et al., 1985). The metabolic pathway undertaken is determined by oxygen concentrations, with anaerobic conditions leading to nitroreduction and aerobic conditions favoring oxidation (El-Bayoumy & Hecht, 1983). Additionally, the metabolic pathways are not exclusive, for example 1-NP can be oxidized and then reduced. Phase II enzymes are then responsible for conjugating the newly reduced and oxidized products with compounds such as glucuronide, glutathione, or sulfate, forming hydrophilic metabolites that are easily excreted (Jancova et al., 2010).

Oxidation

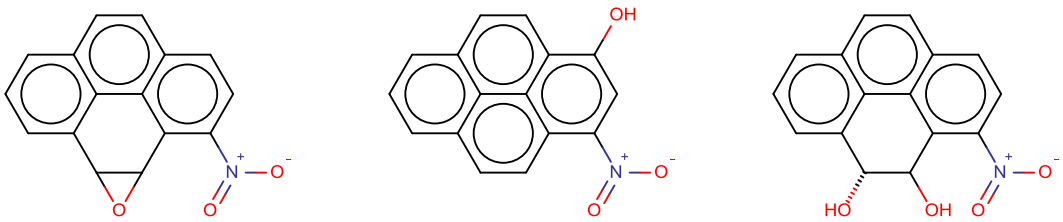

Figure 2. Metabolites formed during oxidation. (L to R) 1-nitropyrene-9,10-oxide, 3-hydroxy-nitropyrene, 1-nitropyrene-trans-9,10-dihydrodiol

Ring oxidation occurs under aerobic conditions, producing a range of hydroxy-1-nitropyrenes as well as K region (the bond between C9 and C10) nitropyrene epoxides (National Toxicology Report, 1996). These epoxides are deleterious as they are capable of interacting with proteins as well as DNA. The epoxides can be further oxidized to form dihydrodiols or conjugated with glutathione or sulfates (National Toxicology Report, 1996). In human liver microsomal samples cytochrome P-450, 3A3 and 3A4 specifically, were identified as the enzymes responsible for 1-NP metabolism, as they were the only class of cytochrome P-450's that exhibited catalytic activity towards 1-NP (Silvers et al., 1992). However, the researchers hypothesized that other classes of P-450 may be involved in 1-NP metabolism because when P-450 3A3 and 3A4 were inhibited roughly 30-40% of the metabolism of 1-NP still occurred. Other studies have been conducted on the metabolism of several mononitropyrenes (1-, 2-, 4-NP) in human breast and immortalized mammary epithelial cells. Cytochrome P-450 1A1 and 1B1 were the identified as the enzymes responsible for formation of metabolites with the potential of reacting with DNA and forming adducts. However, no 1-NP metabolite DNA adducts were identified using HPLC so the it is uncertain if 1-NP is a viable substrate for P-450 1A1 and 1A2 (Sun, Yuan-Wan Guengerich, F P Sharma, Arun Boyiri, Telih Amin,Shantu el Bayoumy, Karam, 2004).

Nitroreduction

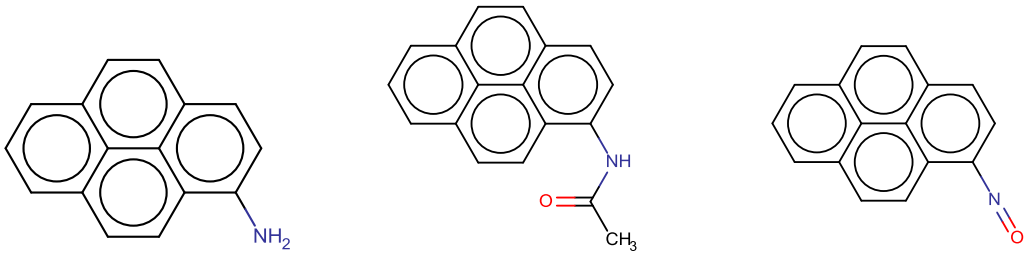

Figure 3. Metabolites formed during nitroreduction: (L to R) 1-aminopyrene, *N*-acetyl-1-aminopyrene, 1-nitrosopyrene

Nitroreduction of 1-nitropyrene has been observed *in vivo* and *in vitro* in a variety of animal systems. The reduction of 1-NP is sequential and the metabolites formed in the process exhibit a broad range of properties. Metabolites such as 1-aminopyrene (1-AP) are less toxic than 1-NP and do not appear to be mutagenic (Manning et al., 1986). However, intermediates formed during the reduction of 1-NP to 1-AP, such as N-hydroxy-1-aminopyrene and 1-nitrosopyrene can react with DNA and proteins (King et al., 1990). *Salmonella typhimurium* served as one on the initial model organisms in which 1-NP metabolites were observed forming DNA adducts (Messier et al., 1981). Later research demonstrated that other cell lines including mammalian cells can form DNA adducts during the nitroreduction of 1-NP (Edwards et al., 1986). Human umbilical cord vein endothelia exposed to low levels of 1-NP display DNA damage, increased production of reactive oxygen species and the up-regulation a stress chaperone protein. However, when nitroreductase activity was inhibited levels of DNA damage and ROS were significantly decreased (Andersson et al., 2009).

Enteric bacteria are believed to have a substantial role in the metabolism of 1-NP in mammals. Human, mouse and rat intestinal flora have all been shown to reduce 1-NP to 1-aminopyrene as well as other minor nitroreduction products (King et al., 1990). Furthermore the formation of 1-NP metabolites, primarily 1-AP, is greatly reduced in antibiotic-treated or germfree animals.

Variations in the metabolites present in germ free and conventional rats treated with 1-NP via oral gavage have been examined. Unlike the conventional rats, no detectable amount of 1-AP were identified in the feces of germ free rats, indicating that the nitroreductase activity of bacteria is involved in 1-NP metabolism (El-Bayoumy et al., 1983). A separate study compared the formation of 1-NP metabolites as well as the macromolecular binding of metabolites in the lung and liver in antibiotic treated rats and wild type rats. Antibiotic treated rats displayed significantly lower macromolecular covalent binding in the lung when compared to control animals. Additionally, control animals displayed greater amounts of 1-AP and 1-acetyaminopyrene in their cecum (a pouch at the start of the large intestine) than the treatment group (Ayres et al., 1985). Intestinal flora is also believed to be involved in the secondary reabsorption of 1-NP metabolites after primary biliary excretion (Medinsky et al., 1985). Furthermore, human fecal bacteria treated with 1-NP are capable of reduction to 1-AP, an indication that unaltered 1-NP can still be reduced within the lower gastrointestinal tract. These studies point to the complex relationship that mammalian microbiota is believed to play in 1-NP metabolism.

Human research

Research into 1-nitropyrene exposure in humans has been limited but is growing. Initial studies examined the response of specific cells line to 1-NP exposure and identified 1-NP and various 1-NP metabolites, including 1-nitrosopyrene as mutagenic and cytotoxic (Patton et al., 1986). Other researchers examined effects of exposure and have tried to identify biomarkers. One proposed biomarker was the nitropyrene hemoglobin adduct. However, a comparison of 1-nitropyrene hemoglobin adduct levels across high, medium and low exposure groups did not find significant difference between adduct concentrations (Zwirner-Baier & Neumann, 1999). This

may have been a result of the half-life of hemoglobin adducts as they are generally stable over the course of an erythrocytes lifespan, roughly 120 days (Franco, 2009).

Several recent studies have quantified other 1-NP metabolites in humans. Toriba et al. (2007) were the first to identify the group of 1-NP metabolites, hydroxy-1-nitropyrenes and hydroxy-*N*-acetyl-1-aminopyrenes in human urine from healthy subjects (Toriba et al., 2007). Expanding on the use of nitropyrene metabolites as biomarkers Miller-Schulze et al. (2013) examined 1-NP metabolites in urine among taxi drivers in China, before, during and after work shifts. Of the metabolites measured, 6- and 8-hydroxy-1-nitropyrene and 8-hydroxy-*N*-acetyl-1-aminopyrene were consistently identified and in concentrations great enough to be quantified. However, metabolite concentrations did not vary significantly between samples collected after work shifts or after time off (Miller-Schulze et al., 2013). The authors estimated the elimination half-life for 6- and 8-hydroxy-1-nitropyrene and 8-hydroxy-*N*-acetyl-1-aminopyrene ranged from 10-12 hours, however variations between populations, individual physiology, and dose represent barriers to the general applicability of this half-life (Miller-Schulze et al., 2013). Another recent study compared 1-NP metabolites and 1-NP exposure between individuals who commute across the U.S.-Mexico border for work versus non-commuters (Galavis, 2013). The study looked specifically at two metabolites of 1-NP, 8-hydroxy-1-nitropyrene (8-OHNP) and 8-hydroxy-*N*-acetyl-1-aminopyrene (8-OHNAAP). When adjusted for creatinine levels, 8-OHNP and 8-OHNAAP concentrations obtained from border commuters were on average twice as high as samples obtained from non-border commuters. Furthermore, a significant relationship was observed between 1-NP exposure as measured by personal air monitors and 8-OHNAAP metabolite concentrations.

A separate metabolite, 1-aminopyrene (1-AP) has also been measured in individuals exposed to clean air and exposed to filtered diesel exhaust. 1-AP concentrations were significantly greater after exposure to diesel exhaust than controls. However, large interpersonal variation existed across urine samples in terms of 1-AP concentration as well as the time from exposure that 1-AP concentration peaks were observed and the duration of detectable 1-AP in urine (Laumbach et al., 2009). Analysis of the observed times of maximum excretion or Tmax of 1-AP appears to identify variance in populations but is unable to identify specific cause for such variance. Two separate groups were observed with distinct excretion patterns. In one group the average Tmax was 5.3 hours with a range between 2 and 10 hours; in the other group the average excretion values grew significantly as samples approached the end of the 24 hour collection window which prevented an accurate Tmax calculation (Huyck et al., 2010). Considering the role intestinal flora, possible subject exposure to diesel engine exhaust outside of the research protocol, as well as variations in metabolism and physiology, this experimental Tmax may not be applicable to other populations or dosages (Huyck et al., 2010).

These previous studies have identified several 1-NP metabolites, dominated by isomers of hydroxy-1-nitropyrenes and hydroxy-*N*-acetyl-1-aminopyrenes (National Toxicology Report, 1996; Toriba et al., 2007). However, much remains to be determined regarding the effects of exposure to 1-NP. Successful quantification of these metabolites would enable researchers to evaluate overall 1-nitropyrene exposure and use this measure as a proxy for diesel exhaust particulate matter exposure. Furthermore, the development and validation of biomarkers is vital for the identification of metabolite half-lives and excretion patterns. The goal of the current study is to develop an accurate and precise a method for quantifying 1-nitropyrene metabolites in urine for use as a biomarkers of DPM exposure.

CHAPTER II

METHODS

Materials

Reference standards were synthesized according to Toriba et al. (2007) and purified by high pressure liquid chromatography (HPLC). Similarly, deuterated metabolites were prepared for use as internal standards. Sep-Pak cartridges were purchased from Waters (Milford, MA). HPLC-grade solvents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All of the experiments were done using filtered and deionized water (18.2 MΩ.cm) (Millipore, Milford, MA). All other chemicals and reagents were purchased commercially at the highest degree of purity available.

Synthesis of reference and deuterated standards

Pyrene was treated with lead tetraacetate in benzene/acetic acid (9:1) and refluxed for 6 hour at 80°C. The resulting acetoxypyrene mixture was purified using silica gel chromatography with a mixture of benzene and hexane as the eluent. The eluate was cooled in an ice bath and then recrystallized using vacuum filtration. The product was then treated with concentrated nitric acid in acetic acid, followed by sodium methoxide in methanol/THF resulting in a mixture of 3-, 6- and 8-OHNP. The OHNPs were separated using HPLC. Portions of the OHNP's were treated with zinc in methanol/Tris-HCL buffer at 90°C and then treated with acetic anhydride in ethyl acetate resulting in various acetoxy-N-acetylaminopyrenes. These were hydrolyzed by treatment with sodium methoxide to produce a mixture of 3-, 6-, and 8-OHNAAPs. The OHNAAPs were purified via HPLC. The deuterated standards were prepared according to the same procedure. The identities of all synthesized compounds were verified confirmed using Acuity Ultra

Performance Liquid Chromatography (UPLC) (Waters, Milford, MA) in conjunction with Aquity Tandem Quadruple (TQ) mass detector (Waters, Milford, MA).

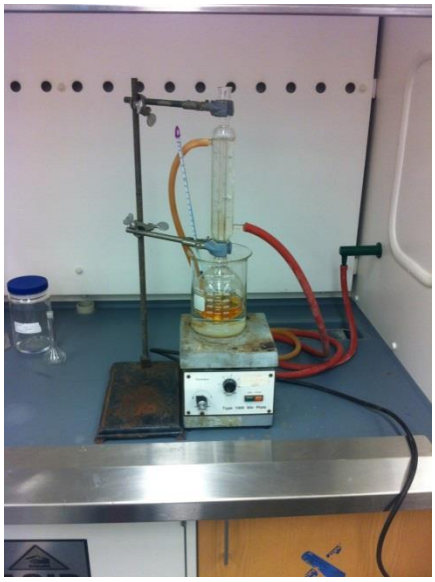

Figure 4. Refluxing of pyrene and lead tetraacetate

Figure 5. UPLC/MS

CHAPTER III

RESULTS

Three of the 1-nitropyrene metabolites, specifically 3-, 6-, and 8-OHNP were successfully synthesized. Total yield of OHNP's was 0.99 g. Identity was confirmed via UV visible spectrophotometry and mass to charge (m/z) ration from MS/MS spectra. Additional confirmation is in progress.

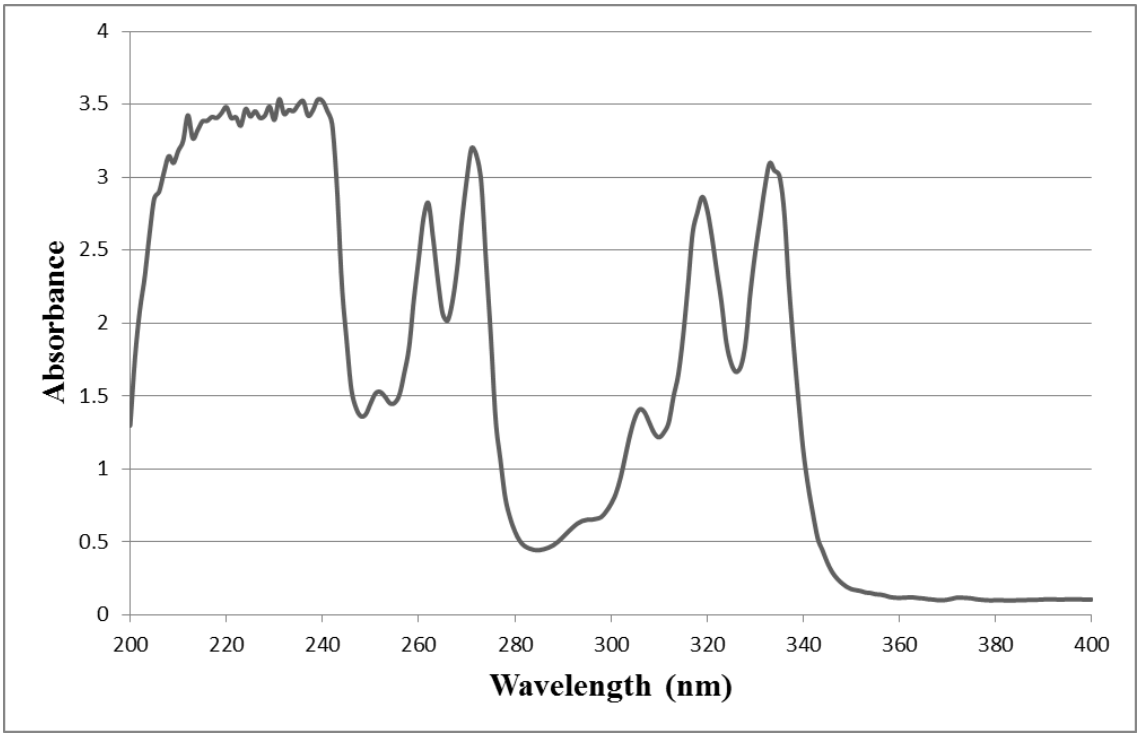

Figure 6. UV visible spectra of synthesized hydroxynitropyrenes prior to separation

Compound	$[M - H]^-$	$[M - NO - H]^-$	$[M - NO_2 - H]^-$
3-OHNP	262.1	232.1	216.1
6-OHNP	262.1	232.1	216.1
8-OHNP	262.1	232.1	216.1

Table 1. MS/MS mass/charge ratio (m/z) of various ions from mass spectrometer

CHAPTER IV

DISCUSSION

The results of this study illustrate the successful development of an analytical method to synthesize, identify and quantify 1-nitropyrene metabolites. The synthesis of 1-NP metabolites is significant, as these will serve as references values to which future experimental samples will be compared. Furthermore, the method of synthesis for metabolites is identical for the deuterated standards. The deuterated standards offer a valuable internal standard that ensures experimental data reliability and will be instrumental for quality control in later research. The findings from this study will assist in future research examining diesel particulate matter exposure. Specifically, this method will be applied on the US-Mexico border. There, diesel exposure will be compared between populations in McAllen, Texas and Monterrey, Mexico. Air pollution ranks among the worst environmental issues in the U.S.-Mexico border region. Spanning almost 2,000 miles, this region represents one of the fastest growing areas in both countries. The border region is home to large scale agricultural operations which contribute to DPM emissions. Furthermore, the rapid growth in the region has led to greater vehicle use, increasing overall emissions. Motorists and commercial trucks often idle for hours as they pass through security checkpoints at ports of entry. Maquiladoras, Mexican assembly plants, are a major source of employment on the border and contribute to the poor air quality with emissions produced during the manufacturing process. The investigation of diesel exhaust exposure on the US-Mexico border will be noteworthy in several ways: first, it will represent a new population added to the limited but growing literature on 1-NP urinary metabolite concentrations; second, the population examined will specifically be pregnant mothers. The collection of urinary metabolites of 1-NP from pregnant mothers is a novel application and will be used to investigate *in utero* exposure of

diesel exhaust. The investigation of *in utero* early childhood exposure is critical as diesel exhaust has been associated with a range of childhood health issues including increased respiratory infection, decreased lung capacity and asthma, which is the most common chronic condition of childhood (Clark et al., 2010; World Health Organization, 2013a).

REFERENCES

Andersson, H., Piras, E., Demma, J., Hellman, B., & Brittebo, E. (2009). *Low levels of the air pollutant 1-nitropyrene induce DNA damage, increased levels of reactive oxygen species and endoplasmic reticulum stress in human endothelial cells*

Atkinson, R., & Arey, J. (1994). Atmospheric chemistry of gas-phase polycyclic aromatic hydrocarbons: Formation of atmospheric mutagens. *Environ.Health Perspect.* **102 Suppl 4**, 117-126.

Ayres, P. H., Sun, J. D., & Bond, J. A. (1985). Contribution of intestinal microfloral metabolism to the total macromolecular covalent binding of 1-nitro-pyrene in the lung and liver of the rat. *Toxicology* **36**(2-3), 263-273.

Bamford, H. A., Bezabeh, D. Z., Schantz, M. M., Wise, S. A., & Baker, J. E. (2003). Determination and comparison of nitrated-polycyclic aromatic hydrocarbons measured in air and diesel particulate reference materials. *Chemosphere* **50**(5), 575-587.

Benbrahim-Tallaa, L., Baan, R. A., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., Guha, N., Loomis, D., & Straif, K. (2012). Carcinogenicity of diesel-engine and gasoline-engine exhausts and some nitroarenes. *The Lancet Oncology* **13**(7), 663-664.

Clark, N. A., Demers, P. A., Karr, C. J., Koehoorn, M., Lencar, C., Tamburic, L., & Brauer, M. (2010). Effect of early life exposure to air pollution on development of childhood asthma. *Environ.Health Perspect.* **118**(2), 284-290.

Edwards, M. J., Batmanghelich, S., Edwards, S., Parry, J. M., & Smith, K. (1986). The induction of DNA adducts in mammalian cells exposed to 1-nitropyrene and its nitro-reduced derivatives. *Mutagenesis* **1**(5), 347-352.

El-Bayoumy, K., & Hecht, S. S. (1983). Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. *Cancer Res.* **43**(7), 3132-3137.

El-Bayoumy, K., Sharma, C., Louis, Y. M., Reddy, B., & Hecht, S. S. (1983). The role of intestinal microflora in the metabolic reduction of 1-nitropyrene to 1-aminopyrene in conventional and germfree rats and in humans. *Cancer Lett.* **19**(3), 311-316.

Franco, R. S. (2009). The measurement and importance of red cell survival. *Am.J.Hematol.* **84**(2), 109-114.

Galavis, G. E. (2013). *Evaluation of exposures to diesel particulate matter utilizing ambient air monitoring and urinary biomarkers among pedestrian commuters who cross the U.S.-Mexico border at San Ysidro, CA.* University of Washington School of Public Health). *Doctoral Dissertation,* , 1-179.

Hayakawa, K., Kizu, R., & Ando, K. (1999). Study on atmospheric behavior and toxicity of carcinogenic nitroarenes by high-performance liquid chromatography using chemiluminescence detection. *Chromatogr J Sep Detect Sci* **20**(1), 37-43.

Howard, P. C., Flammang, T. J., & Beland, F. A. (1985). Comparison of the in vitro and in vivo hepatic metabolism of the carcinogen 1-nitropyrene. *Carcinogenesis* **6**(2), 243-249.

Huyck, S., Ohman-Strickland, P., Zhang, L., Tong, J., Xu, X., & Zhang, J. J. (2010). Determining times to maximum urine excretion of 1-aminopyrene after diesel exhaust exposure. *Journal of Exposure Science and Environmental Epidemiology* **20**(7), 650-655.

International Agency for Research on Cancer. (1989). *1-nitropyrene.* (IARC Monographs Volume 46). Lyons, France: IARC.

International Agency for Research on Cancer. (2012). *IARC: Diesel engine exhaust carcinogenic.* (No. N213). Lyon, France: IARC.

Jancova, P., Anzenbacher, P., & Anzenbacherova, E. (2010). Phase II drug metabolizing enzymes. *Biomed.Pap.Med.Fac.Univ.Palacky Olomouc Czech.Repub.* **154**(2), 103-116.

Kielhorn, J., Wahnschaffe, U., Mangelsdorf, I., & International Programme on Chemical Safety. (2003). *Selected nitro and nitro-oxy-polycyclic aromatic hydrocarbons.* (Environmental Health Criteria 229). Geneva: World Health Organization.

King, L. C., Kohan, M. J., George, S. E., Lewtas, J., & Claxton, L. D. (1990). Metabolism of 1-nitropyrene by human, rat, and mouse intestinal flora: Mutagenicity of isolated metabolites by direct analysis of HPLC fractions with a microsuspension reverse mutation assay. *J.Toxicol.Environ.Health* **31**(3), 179-192.

Laumbach, R., Tong, J., Zhang, L., Ohman-Strickland, P., Stern, A., Fiedler, N., Kipen, H., Kelly-McNeil, K., Lioy, P., & Zhang, J. (2009). Quantification of 1-aminopyrene in human urine after a controlled exposure to diesel exhaust. *J.Environ.Monit.* **11**(1), 153-159.

Lim, S. S., Vos, T., Flaxman, A. D., Danaei, G., Shibuya, K., Adair-Rohani, H., Amann, M., Anderson, H. R., Andrews, K. G., & Aryee, M. (2013). A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: A systematic analysis for the global burden of disease study 2010. *The Lancet* **380**(9859), 2224-2260.

Manning, B. W., Cerniglia, C. E., & Federle, T. W. (1986). Biotransformation of 1-nitropyrene to 1-aminopyrene and N-formyl-1-aminopyrene by the human intestinal microbiota. *J.Toxicol.Environ.Health* **18**(3), 339-346.

Medinsky, M. A., Shelton, H., Shelton, J. A., & McClellan, R. O. (1985). Biliary excretion and enterohepatic circulation of 1-nitropyrene metabolites in fischer-344 rats. *Biochem.Pharmacol.* **34**(13), 2325-2330.

Messier, F., Lu, C., Andrews, P., McCarry, B. E., Quilliam, M. A., & McCalla, D. R. (1981). Metabolism of 1-nitropyrene and formation of DNA adducts in salmonella typhimurium. *Carcinogenesis* **2**(10), 1007-1011.

Miller-Schulze, J. P., Paulsen, M., Kameda, T., Toriba, A., Tang, N., Tamura, K., Dong, L., Zhang, X., Hayakawa, K., & Yost, M. G. (2013). Evaluation of urinary metabolites of 1-nitropyrene as biomarkers for exposure to diesel exhaust in taxi drivers of Shenyang, China. *Journal of Exposure Science and Environmental Epidemiology* **23**(2), 170-175.

National Toxicology Report. (1996). *NTP technical report on toxicity study of 1-nitropyrene administered by inhalation to F333/N rats.* (No. 96-3383). Research Triangle Park, NC: National Institute of Health.

Patton, J. D., Maher, V. M., & McCormick, J. J. (1986). Cytotoxic and mutagenic effects of 1-nitropyrene and 1-nitrosopyrene in diploid human fibroblasts. *Carcinogenesis* **7**(1), 89-93.

Rosenkranz, H. S. (1982). Direct-acting mutagens in diesel exhausts: Magnitude of the problem. *Mutat.Res.* **101**(1), 1-10.

Salmeen, I., Durisin, A. M., Prater, T. J., Riley, T., & Schuetzle, D. (1982). Contribution of 1-nitropyrene to direct-acting Ames assay mutagenicities of diesel particulate extracts. *Mutation Research Letters* **104**(1), 17-23.

Scheepers, P. T., Micka, V., Muzyka, V., Anzion, R., Dahmann, D., Poole, J., & Bos, R. P. (2003). Exposure to dust and particle-associated 1-nitropyrene of drivers of diesel-powered equipment in underground mining. *Ann.Occup.Hyg.* **47**(5), 379-388.

Schuetzle, D., Riley, T. L., Prater, T. J., Harvey, T. M., & Hunt, D. F. (1982). Analysis of nitrated polycyclic aromatic hydrocarbons in diesel particulates. *Anal.Chem.* **54**(2), 265-271.

Silvers, K. J., Chazinski, T., McManus, M. E., Bauer, S. L., Gonzalez, F. J., Gelboin, H. V., Maurel, P., & Howard, P. C. (1992). Cytochrome P-450 3A4 (nifedipine oxidase) is responsible for the C-oxidative metabolism of 1-nitropyrene in human liver microsomal samples. *Cancer Res.* **52**(22), 6237-6243.

Sun, Yuan-Wan Guengerich, F P Sharma, Arun Boyiri, Telih Amin,Shantu el Bayoumy, Karam. (2004). Human cytochromes P450 1A1 and 1B1 catalyze ring oxidation but not nitroreduction of environmental pollutant mononitropyrene isomers in primary cultures of

human breast cells and cultured MCF-10A and MCF-7 cell lines. *Chem.Res.Toxicol.* **17**(8), 1077-85.

Toriba, A., Kitaoka, H., Dills, R. L., Mizukami, S., Tanabe, K., Takeuchi, N., Ueno, M., Kameda, T., Tang, N., & Hayakawa, K. (2007). Identification and quantification of 1-nitropyrene metabolites in human urine as a proposed biomarker for exposure to diesel exhaust. *Chem.Res.Toxicol.* **20**(7), 999-1007.

World Health Organization. (2011). *Exposure to air pollution (particulate matter) in outdoor air.* (No. Fact Sheet 3.3). Copenhagen, Denmark: WHO.

World Health Organization. (2013a). Asthma: Fact sheet. Retrieved 2014, April 4, from http://www.who.int/mediacentre/factsheets/fs307/en/

World Health Organization. (2013b). *The health effects of particulate matter: Policy implications for countries in eastern Europe, Caucasus and Central Asia.* Copenhagen, Denmark: WHO Regional Office for Europe.

World Health Organization. (2014a). *Burden of disease from the joint effects of household and ambient air pollution for 2012.* Geneva Switzerland: World Health Organization.

World Health Organization. (2014b). Outdoor air pollution. Retrieved January 27, 2014, from http://www.who.int/phe/health_topics/outdoorair/en/

Zwirner-Baier, I., & Neumann, H. (1999). Polycyclic nitroarenes (nitro-PAHs) as biomarkers of exposure to diesel exhaust. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **441**(1), 135-144.